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Kinetic Properties of Carbonic Anhydrase Prurified from Erythrocytes of Rainbow Trout (Oncorhynchus mykiss)

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Abstract: Kinetic behavior and some properties of carbonic anhydrase enzyme purified from erythrocytes of rainbow trout (*Oncorhynchus mykiss*) were described at +4°C. The purification procedures were composed of hemolysate preparation, sepharose-4B-L-tyrosine-sulfanylamide affinity gel chromatography and dialyze. Yield and specific activity of enzyme were 20.9 % and 422.5 EU/mg proteins respectively. The overall purification was approximately 200-fold. To check the purity of the enzyme, SDS polyacrylamide gel electrophoresis was performed, which showed a single band. The molecular mass of native enzyme was estimated to be 28,184 kDa by gel filtration column chromatography. Optimal pH, stable pH and optimal temperature of the enzyme were 11.5 in 0.025 M boric acid buffer, 8.5 in 0.025 M Tris-SO_4 buffer and 25aC respectively. K_M and V_{max} values were determined for p-nitrophenylacetate, as a substrate. The inhibitor effect of acetazolamide was examined. The means of activity%-[inhibitor] graph and Lineweaver-Burk graph was used in determination of I_{50} valueand K_i vlue nd the type of inhibition.

Keywords: Fish, erythrocyte, crbonic nhydrse nd chrcteriztion

INTRODUCTION

Concentrtion of crbon dioxide, highly soluble in both queous solution and lipids, is seldom high and it is always in equilibrium with bicarbonate, carbonic acid and carbonate in environments. However, bicarbonate is highly soluble in aqueous solutions but poorly soluble in lipids. Considering these chemical properties, Smith and Ferry (2000) reported that carbon dioxide could freely diffuse in and out of the celland bicarbonate must be transported across the cell membrane.

Perry, (1986) reported that tissue carbon dioxide is diffused into erythrocytes in fish. It is catalyzed to bicarbonate and proton by carbonic anhydrase in erythrocytes. Hemoglobin buffers generated protons whereas most of the bicarbonate anions are passively transported out of the cell in exchange for plasma chloride. Most of the carbon dioxide is thus carried as plasma bicarbonate from the tissues to the gill where the cycle is essentially reversed at the capillaries. Erythrocyte carbonic anhydrase enzyme plays important role in blood carbon dioxide transport process.

Carbonic anhydrase (CA) (carbonate hydrolyase, EC 4.2.1.1) is a member of zinc metalloenzymes. Metalloenzymes are an important class of enzymes used to regulate CO_2 levels in the living organisms. CA catalyses the reversible hydration/dehydration reactions of CO_2 by favoring a different pathway that does not involve the formation of carbonic acid (Beydemir *et al.*, 2000).

The only known physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-; they therefore play key roles in diverse processes, such as physiological pH control and gas balance, calcificationand photosynthesis (Henry and Swenson, 2000; Smith and Ferry, 2000).

A number of 14 different carbonic anhydrase isozymes were described up to now in different tissues and subcellular fractions. Some of these isozymes are cytosolic (such as CA I, CAII, CA III, CA VII), others are membrane-bound (CA IV, CA IX, CA XII and CA XIV), CA V is present only in mitochondria, CA VI is secreted in saliva, whereas several acatalytic forms have also been isolated in recent years (CA VIII, CA X and CA XI) (Supuran and Scozzafava, 2000). These isozymes can be differentiated based on specific activity, subcellular and tissue distribution and their sensitivities to certain inhibitors (Henry *et al.*, 1993).

In most vertebrates, CA activity in blood is restricted to the erythrocytes. The absence of CA activity in blood plasma is thought to be due to the presence of an

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endogenous plasma CA inhibitor (Henry *et al.*, 1997). Lower vertebrates have only one cytoplasmic CA isozyme in their erythrocytes (Gervais and Tufts, 1999). Erythrocyte CA in agnathans and elasmobranchs is similar to the slow type I isozyme in both activity and sensitivity to inhibitors (Henry *et al.*, 1993). However, the teleosts have a fast type II CA isozyme in their erythrocytes (Hall and Schraer, 1983).

The majority of studies on CA function involve the localization of the enzyme to specific tissues and cell types within tissues (Henry and Swenson, 2000) and the physiological measurements after treatment with one or more CA inhibitors (Gilmour *et al.*, 2002; Luquet *et al.*, 1998). But, there are a few studies on characterization of rainbow trout erythrocytes CA in the previous reports without detailed data (Hall and Schraer, 1983).

In the present report we described the purification of carbonic anhydrase from rainbow trout erythrocytes using Sepharose-4B-L tyrosine-sulfanylamide affinity column and expressed different optimal conditions as molecular weight and kinetic behaviors.

MATERIALS AND METHODS

Chemicals: Sepharose 4B, protein assay reagentsand chemicals for electrophoresis were purchased from Sigma-Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Para-aminobenzene sulfanylamide and L-tyrosine were from E.Merck (Merck KGaA Frankfurter strasse 250, D-64293 Darmstadt Germany). All other chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

Purification of carbonic anhydrase isozyme from rainbow trout erythrocytes by affinity chromatography: The fish (250±10 gr. on average) used for the blood samples were obtained from the Fisheries Department, Atatürk University. Having been brought to the biology laboratory of the Department, the fish were first anaesthetized with triciaine methanesulfonate (MS-222); then blood samples were taken from their caudal vein by heparin syringes. The blood samples taken were centrifuged at 1500 rpm for 15 minutes and the plasma and buffy coat were removed. The erythrocyte cells were isolated and washed twice with%0.9 NaCl, they were hemolysed with 1.5 volumes of ice-cold water. For the removal of the ghost and intact cells, hemolysate was centrifuged at 12000 rpm for 45 minutes. The hemolysate pH was adjusted to 8.7 with the solid Tris. It was then applied to the prepared Sepharose 4B-L-tyrosine-sulfanylamide affinity column. The affinity gel was washed with the solution of 25mM Tris-HCl / 22mM Na_2SO_4 (pH 8.7) and the rainbow trout carbonic anhydrase enzyme was eluted with the solution of 0.1 M $NaCH_3COO$/ 0.5 M $NaClO_4$ (pH 5.6). Finally, the eluted enzyme solution was dialyzed against the solution of 0.01 M potassium phosphate/ 0.1 M KCl/ 5mM 2-merkapethanol (pH 7.4). All the above-mentioned studies were carried out at 4aC (Arslan *et al.*, 1996).

Measurement of CA activity: CO_2-hydratase and esterase activities were determined for carbonic anhydrase from rainbow trout erythrocytes. CO_2-hydratase activity of carbonic anhydrase enzyme was assayed colorimetrically using the method of Wilbur and Anderson (1976). CO_2-Hydratase activity as an enzyme unit (EU) was calculated by using the equation (t_0-t_c/t_c) where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. The esterase activity of carbonic anhydrase was determined by the method described by Verpoorte *et al.* (1967). The enzyme activity was determined by following the change in absorbance at 348 nm of p-nitrophenyl acetate to p-nitrophenylate ion over a period of 3 min at 25aC using a spectrophotometer. A blank measurement was obtained by preparing the same cuvette without enzyme solution.

Protein determination: During the purification steps, protein levels were determined spectrophotometrically (595 nm) according to the Bradford method (1976), using bovine serum albumin as the standard. Protein amounts in column fractions were observed via absorbance variations at 280 nm.

Optimal pH determination: For the optimal pH determination, the enzyme esterase activity was measured in 0.025 M Tris-SO_4, 0.025 M phosphate and 0.025 M boric acid buffers within the pH of 7.0 to 9.0, of 5.0 to 7.5 and of 8.0 to 12.0, respectively.

Stable pH Determination: For this purpose, the enzyme esterase activity was determined in 0.025 M Tris-SO_4 buffer in pH of 7.0 to 9.0, 0.025 M phosphate buffer in pH of 5.0 to 7.5 and 0.025 M boric acid buffer in pH of 8.0 to 12.0. In each experiment, the equal volumes of buffer and enzyme solutions were mixed and kept at +4°C. Enzyme activities were determined 24 hr interval for 4 days.

Molecular weight determination: Molecular weight of the native enzyme was determined by the gel filtration in a column of Sephadex G-200 (Demir *et al.*, 2000) with the

following molecular-mass markers; yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa) and horse heart cytochrome C (12.4 kDa) were used as standards (Sigma: MW-GF-200). The void volume was observed with Blue Dextrane (2000 kDa).

The subunit determination of enzyme was made by SDS-PAGE (Laemmli, 1970). The molecular-mass markers; rabbit muscle myosin (205 kDa), $á_2$-macroglobulin (180 kDa), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa) and bovine carbonic anhydrase (29 kDa) were used as standards (Sigma: MW-SDS-200).

Kinetic Studies: At optimum pH and 25aC in order to obtain K_m and V_{max} values for p-nitrophenylacetate substrat, the enzyme activity was measured at five different substrat cuvette concentrations for p-nitrophenylacetate (0.4, 0.6, 0.8, 1 and 1.2 mM). K_m and V_{max} values were determined by means of Lineweaver-Burk graphs. Additionally, Lineweaver-Burk curves were used in determination of the inhibitor constant (K_i) value and inhibition type for acetazolamide.

The inhibitor concentration causing up to 50% inhibition (I_{50}) value was determined by the means of activity%-[inhibitor] graph.

RESULTS

The hemolysate obtained from the rainbow trout erythrocytes applied the affinity gel column (1.3 cm^2 x 60 cm and 25 cm gel height)and then effluents accumulated at an elution speed of 30 mL/hour and as 5 mL-fractions. For the samples in each tube, qualitative protein determination was carried out at 280 mm. The purification process of rainbow trout erythrocytes CA is summarized in Table 1.

SDS polyacrylamide gel electrophoresis was performed after the purification of the enzymeand the electrophoretic pattern was photographed (Fig. 1). At the end of Sephadex G-200 gel filtration column chromatography made for the molecular weight of the active enzyme form, the highest activity was determined in the 52nd tubeand its K_{av} value was found as 0.762. The molecular weight of the native enzyme was approximately estimated to be 28,184 Da (Fig. 2).

The purified enzyme activity was measured in 0.025 M Tris-HCl and 0.025 M phosphate and 0.025 M boric acid buffers at various pH values. CA enzyme was found to be basic character esterase activity was quite high between pH-8 and pH12 in the basic situation. Maximum activity of CA was observed approximately at 11.5 in 0.025 M boric acid buffer (Fig. 3).

Carbonic anhydrase was incubated for 4 days in 0.025 M Tris-SO_4 (pH 7.0-9.0) and 0.025 M phosphate (5.0-7.5) and 0.025 M boric acid (8.0-12.0) buffers at +4aC and enzyme activity was measured at every 24 hr in order to determine the stable pH. The stable pH of the enzyme was determined as 8.5 in 0.025 M Tris-SO_4 buffer (Fig. 4). Furthermore, the enzyme was seen to shown the highest activity at 25aC (Fig 5) after tried between 0-60°C.

I_{50} value of acetazolamide was determined as 0.554 μM by means of Activity-% [acetazolamide] graph (Table 2 and Fig. 6).

The apparent K_M and V_{max} values of rainbow trout gill CA for p-nitrophenylacetate as a substrate were determined by Lineweaver-Burk graph. A K_M of 2.47 mM and a V_{max} of 0.613 μmol / mg protein x minute were obtained for p-nitrophenylacetate. K_i value was also calculated as 0.503±0.29 μM for acetazolamide from Lineweaver-Burk graph and determined that acetazolamide inhibits the enzyme in a noncompetitively manner (Table 2 and Fig. 7).

TAble 1: PruficAtion scheme of cArbonic İnhydrAse from rAinbow trout erythrocytes

PurificAtion step	Activity (EU/ml)	TotAl volume (ml)	Protein (mg/ml)	TotAl protein (mg)	TotAl Activity (EU)	Specific Activity (EU/mg)	Yield (%)	PurificAtion fİctor
HİemolysİteHİemolysİte	53.8	80	28	2240	4304	1.9	100	1
SephArose-4B	-	-	-	-	-	-	-	-
-L tyrosine-	-	-	-	-	-	-	-	-
sulfAnilAmide	-	-	-	-	-	-	-	-
Affinity chrom	-	-	-	-	-	-	-	-
atography	32.4	15	0.142	2.13	486	15.2	11.3	8
DiAlyze	60	15	0.142	2.13	900	422.5	20.9	222.4

TAble 2: K_M, V_{mAx} And dissociAtion constAnt of the enzyme inhibitor complex (Ki) vAlue obtAined from LineweAver-Burk grAph And vAlue of fifty percent inhibition (I_{50}) obtAined from regression AnAlysis grAph for CA from rAinbow trout erythrocytes.

Source	$K_m^{p-nitrophenylAcetAte}$ (mM)	$V_{mAx}^{p-nitrophenylAcetAte}$(($\mu$mol/mg protein.min)	K_i*(μM)	I_{50}*(μM)	Inhibition Type*
Erythrocytes	2.47	0.613	0.503±0.29	0.554	Noncompetitive

*AcetAzolAmide wAs used As inhibitor for I_{50} vAlue And K_i constAnt

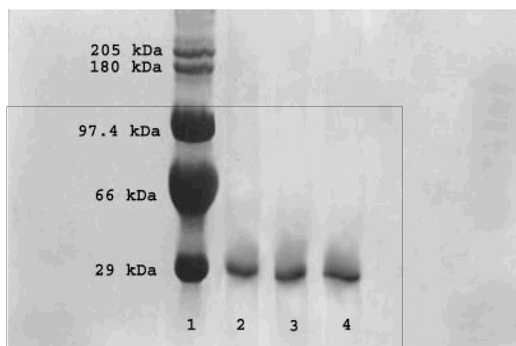

Fig.1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cArbonic anhydrAse purified by affinity gel. Lane 1 shows results for five stAndArd proteins, namely, rabbit muscle myosin (205 kDA), á-macroglobulin (180 kDA), rabbit phosphorylAse B (97.4kDA), bovine serum albumin (66 kDA), and bovine carbonic anhydrase (29 kDA). Lanes 2–4 show results for carbonic anhydrase from an erythrocyte, gill, and lens, respectively, of rainbow trout

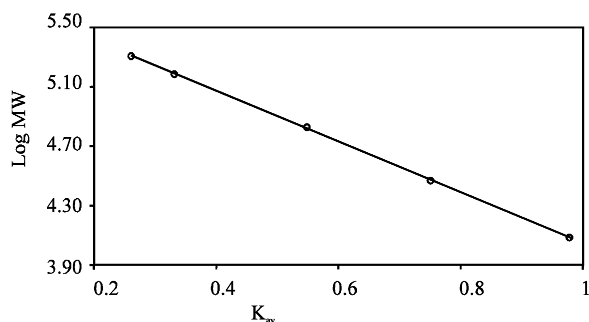

Fig. 2: StandArd K_{Av}-log Molecular Weight graph of CA using gel filtration, (StAndards: Horse heart cytochrome C (12.4 kDA), bovine erythrocyte carbonic anhydrase (29 kDA), bovine serum albumin (66 kDA), yeast alcohol dehydrogenase (150 kDA) and sweet potato β-amylase (200 kDA).

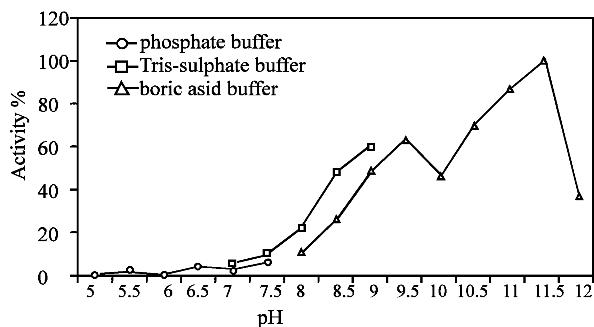

Fig. 3: Activity-pH graph of the rainbow trout erythrocytes

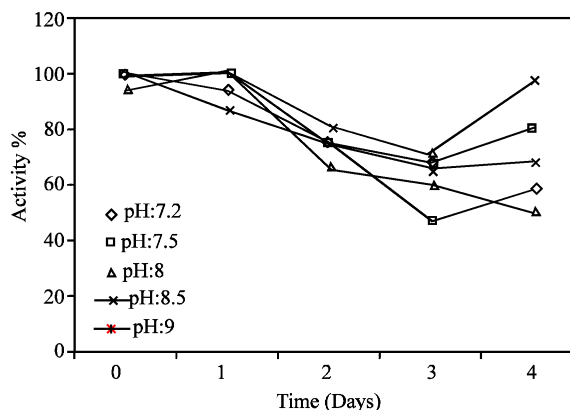

Fig.4: Stable pH graphs of the rainbow trout erythrocytes CA in 1 M Tris SO_4 buffer.

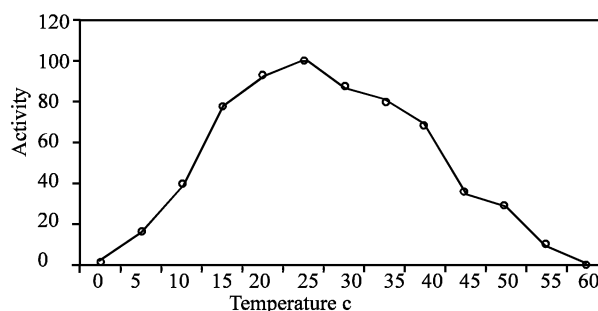

Fig. 5: The effect of the temperature on the rainbow trout erythrocytes CA.

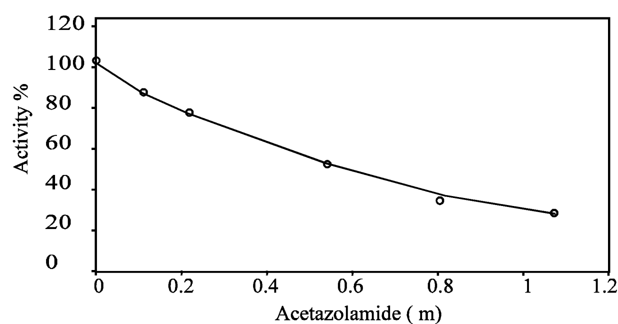

Fig. 6: Results of regressions relating carbonic anhydrase activity in rainbow trout erythrocytes to five different concentrations of acetazolamide

DISCUSSION

Carbonic anhydrase has been purified from many species of animals, plants, yeast and bacteria by using

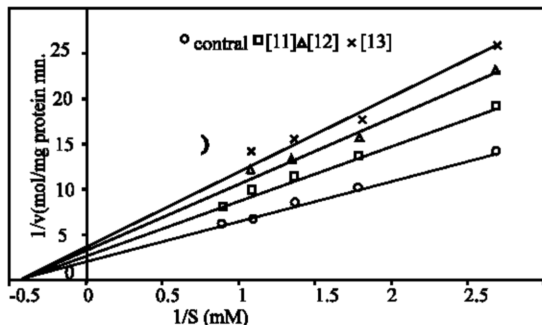

Fig. 7: LineweAver–Burk plots of the inhibition of CA in rAinbow trout erythrocytes by acetazolamide at vArious concentrations. abbreviAtions are as follows: I, inhibitor; V, velocity; and S, substrAte. The controls show reActions with no inhibitor present. It shows the results for acetazolamide ($[I_1]$=0.2 μM, $[I_2]$=0.3 μM and $[I_3]$ =0.45 μM) [noncompetitive inhibition])

different chromatography methods (Feldstein and Silverman, 1984; Burt *et al.*, 1992; Demir *et al.*, 1999). With the use of Sephrarose 4B-L-tirozin-sülfanilamide affinity column in purification of the CA enzyme from the rainbow trout erythrocytes, a higher rate of purification (222.4 times) was ensured through fewer samples (20 mL blood). Hall and Schraer (1983), in their study, could purify 3.7-fold from 300 mL blood sample using the chloroform-ethanol extraction, Sephadex G-75 gel filtration column and DEAE Bio-Gel ion exchange material from the rainbow trout erythrocytes.

Determined molecular weight of erythrocytes CA (approximately 28,000 Da) which had one subunit from rainbow trout was near to that of some other telestean fish species. For instance, Carlsson *et al.* (1980) reported that the molecular weight of the erythrocyte CA enzyme from the cyclostome *Myxine glutinosa* is 29 kDa; Hall and Schaer(1983) reported it to be 28.3 kDa from the trout classified as osteichthyes; Peterson *et al.* (1997)reported it to be 29 kDa from *Danio rerio*.

Testereci . (1999) reported that the activity of carbonic anhydrate enzyme obtained from the gill and liver tissues of the fish in the Van Lake is not affected until pH:7.4and a significant increase in the enzyme activity over pH:8. They concluded that the esterase activity of this enzyme at the high pH was impossible to evaluate as this increase was due to kinetic reaction between substrate (p-nitropheny acetate) and buffer at pH: 8-11.5. Considering this assumption, the kinetic reaction between substrate and basic buffer solutions was eliminated in order to determine the optimum pH by using double-beam spectrophotometer. Therefore both the activity of enzyme and related optimum pH colud be determined in the present study.

The k_{cat} values of the CA-III and CA-V isozymes in physiologic pH=7 are much less than that of such isozymes as CA-II. This low activity is, however, attributed to the fact that they do not have an efficient proton shuttle of pK_a near 7 (Jewell *et al.*, 1991). Furthermore, Qian *et al.* (1999) reported that the presence of enhanced values of k_{cat} for hydration of CO_2 in the region of pH > 8 for carbonic anhydrase isozymes II, III, IV, Vand VII. They also suggested that the increase observed in k_{cat} is due to an enhanced proton transfer at pH > 8 reflecting the presence of residues of basic pK_a that act as proton acceptors. This situation can be accepted as a possible explanation of why rainbow trout erythrocyte CA enzyme had an optimum pH: 11.5 in the present study.

Determined temperature value (25aC) for the maximum CA enzyme activity in fish is considerably different than the other reported temperature values for this purpose i.e. in mammals (36.5aC) (Demir *et al.*, 2000), in plants (70aC) (Demir *et al.*, 1999). The possible reason for these differences might be the species effect and habitat differences (López Maòanes *et al.*, 2000) since every organism has a special optimum temperature for optimum growth and other physiological functions (Aldrich and Saunders, 2001).

I_{50} value of acetazolamide (the heterocyclic sulfonamide) for rainbow trout erythrocyte CA was found as 0.554 μM. I_{50}value of acetazolamide was reported to be 0.2 μM for CA-I; 0.44 μM for CA-II; 0,066 μM for CA-IV (Maren *et al.*, 1993). However, it must also be considered that in obtaining these values, different techniques and different sources were used.

From the Lineweaver-Burk graph, the obtained K_M and a V_{max} values (2.47 mM and 0.613 μmol/mg protein.min.) were similar to those obtained from bowfin erythrocyte, bovine erythrocyte plasma membrane and rat erythrocyte CA-I (Feldstein and Silverman, 1984; Gervais and Tufts, 1999; Demir *et al.*, 2000). K_i value obtained for acetazolamide was similar to *Plasmodium falciparium* and human CA-II (Krungkrai *et al.*, 2001).

It is know that carbonic anhydrate inhibitors interact with zinc ion and the OH and NH groups of aminoacid Thr- 199. and, some other CA enzyme inhibitors can also interact with a hydrophobic area which is made up of amino acid residues and which is located around the Zn (II) ion (Briganti *et al.*, 1998). In the present study, acetazolamide inhibited the enzyme in a noncompetitively manner, therefore as a consequent, the rainbow trout erythrocytes CA enzyme can be classified as a CA II isozymes on the basis of its high specific activity and its sensitivity to acetazolamide inhibition with the 11.5

optimum pH, 25aC optimum temperature and 8.5 stabile pH. Thus, hopefully, the biochemical properties of this enzyme reported here could be the starting point for further studies.

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